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(21) International Application Number: PCT/DK91/00100 (22) International Filing Date: 16 April 1991 (16.04.91) (30) Priority data: 954/90 18 April 1990 (18.04.90) DK (71) Applicants (for all designated States except US): NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsvaerd (DK). LYSI HF [IS/IS]; Grandavegur 42, IS-121 Reykjavik (IS). (72) Inventors; and (75) Inventors/Applicants (for US only) : HARALDSSON, Gudmundur, G. [IS/IS]; Skogaras 11, IS-110 Reykjavik (IS). SVANHOLM, Hanne [DK/DK]; Gladsaxevej 80, DK-2860 Soeborg (DK). HJALTASON, Baldur [IS/IS]; Nordurstigur 3, IS-101 Reykjavik (IS).		(74) Common Representative: NOVO NORDISK A/S; Patent Dept., Novo Allé, DK-2880 Bagsvaerd (DK). (81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent), US. Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: PROCESS FOR PREPARATION OF TRIGLYCERIDE AND TRIGLYCERIDE COMPOSITION		
(57) Abstract <p>Triglyceride wherein all three fatty acids are C₂₀₊ polyunsaturated fatty acids (with at least three double bonds) is prepared by esterification of glycerol with free polyunsaturated fatty acid or its C₁₋₄ lower alkyl ester in the presence of a lipase. The yield of triglyceride is increased and the amount of mono- and diglyceride decreased by removing water or lower alcohol formed during the reaction, by using positionally non-specific lipase, and/or by using a lipase immobilized by adsorption on a particulate, macroporous adsorbent resin of the acrylic type.</p>		

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PROCESS FOR PREPARATION OF TRIGLYCERIDE AND TRIGLYCERIDE COMPOSITION

TECHNICAL FIELD

The invention relates to a process for the preparation of a triglyceride
5 wherein all three fatty acids are C_{20+} polyunsaturated fatty acids (with at least three double bonds), by esterification of glycerol with free polyunsaturated fatty acid or its C_{1-4} lower alkyl ester in the presence of a lipase. The invention also relates to a triglyceride composition with a high content of polyunsaturated fatty acid.

BACKGROUND ART

10 It is known that triglycerides of poly-unsaturated fatty acid (PUFA), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have beneficial medical effects, and within the last decade much attention has been directed to methods of producing triglyceride compositions with a high content of these acids, and particularly a high content of triglycerides with three such acids in the molecule.
15 PUFA in the form of free fatty acid or lower alkyl (e.g. methyl or ethyl) ester is available in high purity and have been used to prepare triglycerides with high PUFA content.

Thus, JP-A 61-43143 (Nisshin Flour Mill et al.) and EP 300,844 (R.F. Azar et al.) describe chemical interesterification of lower alkyl PUFA ester with
20 triacetin or tributyrin. Sodium methylate was used as catalyst, and vacuum was used to remove lower alkyl acetate or butyrate formed in the reaction. The former describes production of triglyceride with 90% PUFA content.

JP-A 61-246146 (Nissui Seiyaku) describes halogenation of PUFA free acid, followed by reaction of PUFA acyl chloride with glycerine. The first step was
25 carried out with oxalyl chloride at 65-90°C for 4 hours, and the second step under reflux for several hours in chloroform in the presence of quinoline or pyridine.

The above-mentioned processes use highly reactive chemicals that require special precautions in handling, these reactive chemicals react with part of the labile PUFA acyl groups, and the resulting reaction mixture in each case requires complex purification.

5 JP-A 62-91188 (Nisshin Oil) describes lipase-catalyzed production of PUFA glycerides from glycerol and PUFA free acid or ethyl ester, using positionally specific lipase in native form or immobilized on a weakly basic anion exchange resin. It is stated that addition of water is necessary. After reaction and removal of unreacted fatty acid, the glyceride mixture contained at most 86% triglyceride
10 together with at least 14% diglyceride + monoglyceride. The product contained at most 85% polyunsaturated fatty acids together with at least 15% of other fatty acids.

It is an object of the invention to provide a simple process, avoiding the use of aggressive chemicals, to produce triglyceride with a low content of mono- and diglycerides, having a high content of PUFA, especially a high content of
15 triglycerides with three PUFA in the molecule. It is also an object to provide triglyceride compositions with high PUFA content.

STATEMENT OF THE INVENTION

We have found that the yield of triglyceride can be increased and the amount of mono- and diglyceride decreased by removing water or lower alcohol
20 formed during the reaction, by using positionally non-specific lipase, or by using a lipase immobilized by adsorption on a particulate, macroporous adsorbent resin of the acrylic type.

Accordingly, the invention provides a process for the preparation of a triglyceride wherein all three fatty acids are C_{20+} polyunsaturated fatty acids (with
25 at least three double bonds), by esterification of glycerol with free polyunsaturated fatty acid or its C_{1-4} lower alkyl ester in the presence of a lipase. In the first aspect of the invention, the process is characterized by removing water or lower alcohol by evaporation during the reaction. In another aspect, the process is characterized in

that the lipase is positionally non-specific. In a third aspect, the process is characterized in that the lipase is immobilized by adsorption on a particulate, macro-porous adsorbent resin of the acrylic type.

The invention also provides a triglyceride composition, characterized by at least 95% by weight (preferably at least 98%) of the fatty acids in the triglyceride molecules being polyunsaturated C₁₈₋₂₂ acid.

DETAILED DESCRIPTION OF THE INVENTION

Polyunsaturated fatty acid

The process of the invention is applicable to C₂₀₊ PUFA with 3 or 10 more double bonds such as eicosapentaenoic acid (EPA, C_{20:5}) and docosahexaenoic acid (DHA, C_{22:6}).

Thus, the process of the invention can be used to prepare triglyceride compositions with high content of PUFA by using a reactant mixture wherein the free acid or lower alkyl ester contains at least 90%, preferably at least 95% and most 15 preferably at least 98%, of PUFA. In this way it is possible to prepare triglyceride with PUFA in all three positions in good yield. In this connection, it has surprisingly been found that pure EPA or DHA is incorporated particularly fast.

Preferably, the PUFA reactant is free fatty acid, methyl or ethyl ester; These are easily available, and the resulting water, methanol or ethanol is very 20 volatile and is easily removed.

The PUFA free fatty acid or ester may be prepared by known methods, and some are commercially available in high purity, e.g. EPA and DHA as free acid and ethyl ester in 99% purity from Idemitsu Petro Chemical Co., Ltd., Japan.

Lipase

25 The lipase should be sufficiently thermostable for the temperature and reaction time in question, e.g. 24 hours at 40-80°C. It is preferred to use immobilized lipase.

One aspect of the invention uses a positionally non-specific lipase. Examples of non-specific lipases are those derived from strains of *Candida*, especially *C. antarctica* lipase (WO 88/02775, incorporated herein by reference), and lipase from *C. rugosa* (also known as *C. cylindracea*). It is particularly preferred to use a lipase preparation containing both lipase A and lipase B of *C. antarctica* described in said reference.

Positionally specific (1,3-specific) lipase may be used in some embodiments of the invention. Examples are lipase derived from *Humicola*, especially *H. lanuginosa* (WO 89/06278) and recombinant *Humicola* lipase (EP 10 305,216) and *Mucor* lipase (EP 140,542).

One aspect of the invention uses lipase immobilized by adsorption on a particulate, macroporous adsorbent (i.e. non-ionic) resin of the acrylic type according to WO 89/02916.

An example of a lipase preparation that may be used is SP 382 from Novo Nordisk A/S (mixture of lipases A and B from *C. antarctica*, immobilized according to WO 89/02916).

Reaction conditions

A suitable amount of lipase is generally in the range 0.5 - 10 BIU/g (typically 1-5 BIU/g) of reactant mixture (BIU = Batch Interesterification Unit, see WO 89/06278) by use of immobilized lipase, or 50 - 500 LU/g of oil (LU = Lipase Unit, see WO 88/02775) by use of native (non-immobilized) lipase.

It is preferable to use the two reactants at about the stoichiometric ratio or with a moderate excess (e.g. 0-50%, especially 0-20%) of the PUFA acid or ester. It is preferable to let the reaction continue until at least 90% (particularly more than 95, especially more than 98%) of the glycerol has been converted into triglyceride.

It is generally not necessary to use a pH buffer or an organic solvent in the process.

A temperature of 40-80°C, especially 60-80°C, is generally suitable for the reaction and the evaporation. The reaction time will generally be from 24 - 48 hours.

Removal of volatile alcohol or water

The removal by evaporation of volatile alcohol or water may be done continuously from a stirred tank. Reactants may be added batch wise, semi-batch wise or continuously. If the lipase is immobilized it can be separated off after the reaction and reused.

Alternatively, the reaction may occur in two or more steps, and evaporation can be done between the steps. Each process step can be made in a stirred tank, or immobilized lipase can be used continuously in a fixed bed.

The evaporation is most conveniently done under vacuum, e.g. below 200 Pa and especially below 20 Pa.

EXAMPLES

EXAMPLE 1

Preparation of triglycerides containing approx. 85% EPA/DHA

Immobilized lipase derived from *Candida antarctica* (SP-382 from Novo Nordisk A/S; activity approx. 30 BIU/g; 1.53g; moisture-free) was added to a mixture of glycerol (99% from Sigma; 1.37g, 14.9 mmol) and 87% PUFA (free acid) concentrate (55% EPA and 32% DHA; M.wt. 311.0 g/mol; 14.1g, 45.3 mmol). The mixture was gently stirred on a magnetic stirrer hot-plate at 65°C under continuous vacuum of 0.1 mm Hg. The volatile water or lower alcohol, when using lower alkyl ester concentrates, produced during the progress of the reaction was continuously condensed into a liquid nitrogen cooled trap, which could be separated and weighed regularly during the process by disconnecting the reaction by replacing the vacuum with dry nitrogen or argon atmosphere. After 30 hours the reaction was discontinued, hexane added and the enzyme separated off by filtration. The hexane was removed in vacuo on a rotary evaporator. Titration was applied to determine the free fatty acid content of the crude reaction product (3% FFA content, corresponding to 97% incorporation, which is equivalent to 91% triglyceride content). The titration results were confirmed by *latroscan* studies, which indicated 91% triglyceride

content, after the product had been freed from free fatty acids by washing the organic phase a few times with 0.25M sodium hydroxide in 1:1 water/ethanol solution. 100% pure triglycerides were afforded by preparatory High Performance Liquid Chromatography (HPLC) eluting with 10% ether in hexane solvent, which was confirmed by latroscan studies. Capillary Gas Liquid Chromatography (GLC) analysis showed fatty acid composition identical to the original PUFA concentrate.

The following equation was used to calculate the % incorporation of fatty acids into glycerol from the weight measurements:

$$\% \text{incorporation} = 1.71 \cdot [\text{Wt}_{\text{water}} / \text{Wt}_{\text{glycerol}}] \cdot 100\%$$

10 This was deduced from the following equation:

$$\begin{aligned} \% \text{incorp.} &= 1/3 \cdot [\# \text{ of eq. of entrapped water} / \\ &\quad \# \text{ of eq. of glycerol used}] \cdot 100\% \\ &= 1/3 \cdot [\text{Wt}_{\text{water}} / \text{M.wt}_{\text{water}}] / [\text{Wt}_{\text{glycerol}} / \text{M.wt}_{\text{glycerol}}] \cdot 100\% \\ &= 1/3 \cdot [\text{Wt}_{\text{water}} / \text{Wt}_{\text{glycerol}}] \cdot [\text{M.wt}_{\text{glycerol}} / \text{M.wt}_{\text{water}}] \cdot 100\% \end{aligned}$$

$$15 = 1/3 \cdot [\text{Wt}_{\text{water}} / \text{Wt}_{\text{glycerol}}] \cdot [92.1 / 18.0] \cdot 100\%$$

$$= 1.71 \cdot [\text{Wt}_{\text{water}} / \text{Wt}_{\text{glycerol}}] \cdot 100\%$$

The following results were obtained:

Time hours	% incorporation*	
	min.*	max.*
1	50	56
2	67	72
25 6	90	96
24	103**	108
30	103**	108

- * The incorporation was determined by mass measurements of the entrapped product. The minimum incorporation was based on the assumption that all the moisture had escaped from the immobilized lipase.
- ** Due to inaccuracy in the mass measurements the minimum incorporation passes the 100% incorporation level.

EXAMPLE 2

Preparation of triglycerides containing approx. 60-70% EPA/DHA

The procedure of Example 1 was followed in details by using immobilized lipase SP-382 (1.10g; 10% moisture content), which was added to a mixture of glycerol (1.00g, 10.9 mmol) and 66% PUFA concentrate (38% EPA and 28% DHA; M.wt. 309.5 g/mol; 10.2g, 33.0 mmol) or 59% PUFA concentrate (29% EPA and 30% DHA; M.wt. 306.4 g/mol; 10.1 g; 33.0 mmol).

The following results were found:

Time, hours	% incorporation*			
	59% PUFA		66% PUFA	
	min.	max.	min.	max.
1	53	72	34	53
2	67	85	61	80
3	77	96	73	92
6	89	108	79	97
24	106**	125	90	109
28	106**	125	90	109

- * The incorporation was determined by mass measurements of the entrapped product. The minimum incorporation was based on the assumption that all the moisture had escaped from the immobilized lipase.
- ** Due to inaccuracy in the mass measurements the minimum incorporation passes the 100% incorporation level.

EXAMPLE 3

Preparation of triglycerides containing 99% EPA

Immobilized lipase SP 382 (0.50 g; moisture-free) was added to a mixture of glycerol (99% from Sigma; 0.44 g, 4.78 mmol) and 99% EPA as free fatty acids (M.wt. 302.5 g/mol; 4.40 g, 14.54 mmol). The mixture was gently stirred on a magnetic stirrer hot-plate at 65°C under continuous vacuum of 0.5 - 0.1 mmHg. The volatile water produced during the progress of the reaction was continuously condensed into a liquid nitrogen cooled trap, which could be separated and weighed regularly during the process by disconnecting the reaction by replacing the vacuum with dry nitrogen or argon atmosphere. After 30 hours the reaction was discontinued, hexane added and the enzyme separated off by filtration. The organic solvent was removed in vacuo on a rotary evaporator to afford the crude product as a slightly yellowish oil (4.18 g, 93%). Weight measurements indicated 105 - 108% incorporation, but NMR spectroscopy indicated 98% incorporation, which had increased to 99% after 48 hours. Titration was applied to determine the free acid content of the crude reaction product (less than 1% FFA content, corresponding to at least 99% incorporation, which is equivalent to 97% triglyceride content). The crude product was directly introduced into HPLC eluting with 10% ether in hexane solvent to afford 100% pure triglycerides (3.51 g, 84% recovery from HPLC, but overall yield 78%) which was confirmed by iatrosan studies.

[250 MHz ^1H NMR (CDCl_3): δ 5.41-5.26 (m, 31 H, =C-H and -CH₂-CH-CH₂-), 4.30 (dd, J = 11.90 Hz, J = 4.34 Hz, 2 H, -CH₂-CH-CH₂-), 4.14 (dd, J = 11.90 Hz, J = 5.93 Hz, 2 H, -CH₂-CH-CH₂-), 2.90-2.78 (m, 24 H, =C-CH₂-C=), 2.33 (t, J = 7.34 Hz, 2H, OOC-CH₂-), 2.32 (t, J = 7.36 Hz, 4 H, OOC-CH₂-), 2.15-2.01 (m, 12 H, -CH₂-CH₂-C=), 1.75-1.61 (m, 6H, =CH-CH₂-CH₃), and 0.97 ppm (t, J = 7.52 Hz, 9 H, -CH₃). ^{13}C NMR (CDCl_3): δ 172.9(s), 172.6(s), 132.0(d), 128.9(d), 128.7(d), 128.5(d), 128.2(d), 128.1(d), 128.1(d), 128.0(d), 127.8(d), 127.0(d), 68.9(d), 62.1(t), 33.5(t), 33.3(t), 26.4(t), 25.6(t), 25.6(t), 25.5(t), 24.7(t), 24.6(t), 20.5(t) and 14.2 ppm(q), IR (neat liquid): ν_{max} 3020 (vs, C=C-H), 2970 (s, CH₃), 2935 (s, CH₂), 2875 (s, CH₃), 2850 (w, CH₂), 1745 (vs, C=O) and 1645 cm⁻¹ (ms, C=C). m/e (EI): 945

(M^+ , 100%); found 944.68784 $C_{63}H_{92}O_6$ requires 944.68939 amu].

The following results were obtained:

5	Time hours	% incorporation		
		min.*	max.*	NMR
	1	56	62	60
10	2	72	78	74
	4	91	97	89
	6	92	98	91
	12	95	101	96
	24	99	105	97
15	30	99	105	98
	48	99	105	99

- * The incorporation was determined by mass measurements of the entrapped product. The minimum incorporation was based on the assumption that all the moisture had escaped from the immobilized lipase.

20 EXAMPLE 4

Preparation of triglycerides containing 99% DHA

Immobilized lipase SP 382 (0.50 g; moisture-free) was added to a mixture of glycerol (99% from Sigma; 0.41 g, 4.45 mmol) and 99% DHA as free fatty acids (M.wt. 328.5 g/mol; 4.43 g, 13.48 mmol). The mixture was gently stirred on a 25 magnetic stirrer hot-plate at 65°C under continuous vacuum of 0.5 - 0.1 mmHg. The volatile water produced during the progress of the reaction was continuously condensed into a liquid nitrogen cooled trap, which could be separated and weighed regularly during the process by disconnecting the reaction by replacing the vacuum with dry nitrogen or argon atmosphere. After 30 hours the reaction was 30 discontinued, hexane added and the enzyme separated off by filtration. The organic

solvent was removed in vacuo on a rotary evaporator to afford the crude product as a slightly yellowish oil (4.36 g, 95%). Weight measurements indicated 100 - 106% incorporation which remained constant from 24 to 72 hours, whereas NMR spectroscopy indicated 97% incorporation after 24 hours which had increased to 100% after 72 hours. The crude product was directly introduced into HPLC eluting with 10% ether in hexane solvent to afford pure triglycerides (3.46 g, 80% recovery from HPLC, but overall yield 76%) which was confirmed by iatroscan studies.

[250 MHz ^1H NMR (CDCl_3): δ 5.44-5.25 (m, 37 H, =C-H and -CH₂-CH-CH₂-), 4.30 (dd, J = 11.90 Hz, J = 4.36 Hz, 2 H, -CH₂-CH-CH₂-), 4.15 (dd, J = 11.90 Hz, J = 5.89 Hz, 2 H, -CH₂-CH-CH₂-), 2.90-2.79 (m, 30 H, =C-CH₂-C=), 2.39-2.38 (m, A₂B₂, 12 H, =CH-CH₂-CH₂-COOH), 2.13-2.02 (m, 6 H, =CH-CH₂-CH₃), and 0.97 ppm (t, J = 7.53 Hz, 9 H, -CH₃). ^{13}C NMR (CDCl_3): δ 172.5(s), 172.1(s), 132.0(d), 129.5(d), 128.5(d), 128.3(d), 128.3(d), 128.2(d), 128.2(d), 128.0(d), 127.9(d), 127.8(d), 127.6(d), 127.0(d), 69.0(d), 62.2(t), 34.0(t), 33.8(t), 25.6(t), 25.6(t), 25.6(t), 25.5(t), 22.6(t), 20.5(t) and 14.2 ppm(q), IR (neat liquid): ν_{max} 3020 (vs, C=C-H), 2970 (s, CH₃), 2930 (s, CH₂), 2870 (s, CH₃), 2850 (w, CH₂), 1750 (vs, C=O) and 1650 cm⁻¹ (ms, C=C). m/e (EI): 1023 (M⁺, 100%); found 1022.7340 C₆₉H₉₈O₆ requires 1022.7363 amu].

The following results were obtained:

Time hours	% incorporation		
	min.*	max.*	NMR
25	1	18	24
	2	43	45
	4	59	67
	6	71	77
	12	91	91
30	24	100	97
	30	100	98
	48	100	99
	72	100	100

- * The incorporation was determined by mass measurements of the entrapped product. The minimum incorporation was based on the assumption that all the moisture had escaped from the immobilized lipase.

EXAMPLE 5

5 Preparation of triglycerides with reuse of lipase

Immobilized lipase SP-382 (9.31g; moisture-free) was added to a mixture of glycerol (9.00g; 97.7 mmol) and free fatty acids from cod liver oil (9% EPA and 9% DHA; M.wt. 285.0 g/mol; 84.1g; 295 mmol). The mixture was gently stirred at 65°C under a continuous vacuum of 0.1 mm Hg. The volatile water produced during the progress of the reaction was condensed into a liquid nitrogen cooled trap, which was weighed regularly during the progress of the reaction. After 48 hours the reaction was discontinued and the lipase directly separated off without an organic solvent by filtration under dry nitrogen by the aid of a pressure equalized funnel equipped with a sintered glass filter plate and inlets/outlets to the nitrogen and the vacuum lines to aid the filtration, which was controlled by teflon key stopcocks. The oil was collected for further analysis.

The immobilized lipase was reintroduced into the reaction vessel. This was repeated 5 times, reusing the same immobilized lipase.

The following results were obtained:

20	Run	% incorporation*	
	No.	min.	max.
	1	93	96
25	2	93	97
	3	93	97
	4	93	97
	5	94	98

- * As determined by mass measurements of the entrapped product after 48 hours.

CLAIMS

1. A process for the preparation of a triglyceride wherein all three fatty acids are C_{20+} polyunsaturated fatty acids (with at least three double bonds), by esterification of glycerol with free polyunsaturated fatty acid or its C_{1-4} lower alkyl ester in the presence of a lipase, characterized by removing water or lower alcohol by evaporation during the reaction.
2. A process according to Claim 1, wherein water or lower alcohol is removed under vacuum below 20 Pa, preferably at 40-80°C.
3. A process for the preparation of a triglyceride wherein all three fatty acids are C_{20+} polyunsaturated fatty acids (with at least three double bonds), by esterification of glycerol with free polyunsaturated fatty acid or its C_{1-4} lower alkyl ester in the presence of a lipase, characterized in that the lipase is positionally non-specific.
4. A process according to Claim 3, wherein the lipase is derived from a strain of *Candida*, most preferably *C. antarctica*.
5. A process according to Claim 3 or 4, further characterized as in Claim 1 or 2.
6. A process according to any preceding claim, wherein the lipase is immobilized.

7. A process for the preparation of a triglyceride wherein all three fatty acids are C_{20+} polyunsaturated fatty acids (with at least three double bonds), by esterification of glycerol with free polyunsaturated fatty acid or its C_{1-4} lower alkyl ester in the presence of a lipase, characterized in that the lipase is immobilized by adsorption on a particulate, macroporous adsorbent resin of the acrylic type.

8. A process according to Claim 7, further characterized as in any of Claims 1 - 5.

9. A process according to any preceding claim, wherein the free fatty acid or ester in the reaction mixture is at least 90% pure, preferably at least 95% pure, and most preferably at least 98% pure.

10. A process according to any preceding claim, wherein no buffer or organic solvent is used.

11. A process according to any preceding claim, whereby the reaction time is below 30 hours.

12. A process according to any preceding claim, wherein the polyunsaturated fatty acid is eicosapentaenoic acid, docosahexaenoic acid or a combination of these.

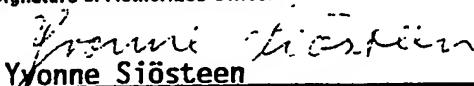
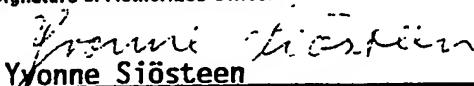
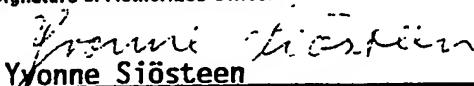
13. A process according to any preceding claim, using a stoichiometric excess of 0-50% (preferably 0-20%) of the fatty acid or ester relative to glycerol.

14. A process according to Claim 13, wherein the reaction is continued until at least 90%, preferably at least 95% and most preferably at least 98% of the glycerol has been converted into triglyceride.

15. A process according to any preceding claim, wherein the reactant free acid or ester is a free fatty acid or a methyl or ethyl ester.
16. A triglyceride composition, characterized by at least 95 % by weight (preferably at least 98%) of the fatty acids in the triglyceride molecules being poly-5 unsaturated C₁₈₋₂₂ acid.
17. A composition according to Claim 16, wherein the polyunsaturated acid is eicosapentaenoic acid, docosahexaenoic acid or a mixture of these.

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 91/00100

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: C 12 P 7/64, C 11 C 3/02, 3/06																	
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%; border-bottom: 1px solid black;">Classification System</th> <th style="border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="height: 40px; vertical-align: bottom; border-right: 1px solid black;">IPC5</td> <td>C 11 C; C 12 P</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched⁸</div> <p>SE,DK,FI,NO classes as above</p>			Classification System	Classification Symbols	IPC5	C 11 C; C 12 P											
Classification System	Classification Symbols																
IPC5	C 11 C; C 12 P																
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹ <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; border-bottom: 1px solid black;">Category *</th> <th style="border-bottom: 1px solid black;">Citation of Document,¹¹ with indication, where appropriate, of the relevant passages¹²</th> <th style="border-bottom: 1px solid black;">Relevant to Claim No.¹³</th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td>GB, A, 2205850 (KANEGAFUCHI KAGAKU KOGYO KABUSHIKI KAISHA) 21 December 1988, see page 1 line 29 - page 2 line 9 --</td> <td style="text-align: center; vertical-align: top;">1-2,6,9-17</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td>Patent Abstracts of Japan, Vol 11, No 300, C449, abstract of JP 62- 91188, publ 1987-04-25 THE NISSHIN OIL MILLS LTD --</td> <td style="text-align: center; vertical-align: top;">1-2,6,9-17</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td>Chemical Abstracts, volume 111, no. 17, 23 October 1989, (Columbus, Ohio, US), see page 580, abstract 152188r, & JP, A, 185089 (Synthesis of triglycerides by immobilized lipase.) 30 March 1989 --</td> <td style="text-align: center; vertical-align: top;">1-2,6,9-17</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">A</td> <td>EP, A1, 0064855 (UNILEVER NV) 17 November 1982, see the whole document --</td> <td style="text-align: center; vertical-align: top;">1-2,6,9-17</td> </tr> </tbody> </table>			Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	Y	GB, A, 2205850 (KANEGAFUCHI KAGAKU KOGYO KABUSHIKI KAISHA) 21 December 1988, see page 1 line 29 - page 2 line 9 --	1-2,6,9-17	Y	Patent Abstracts of Japan, Vol 11, No 300, C449, abstract of JP 62- 91188, publ 1987-04-25 THE NISSHIN OIL MILLS LTD --	1-2,6,9-17	Y	Chemical Abstracts, volume 111, no. 17, 23 October 1989, (Columbus, Ohio, US), see page 580, abstract 152188r, & JP, A, 185089 (Synthesis of triglycerides by immobilized lipase.) 30 March 1989 --	1-2,6,9-17	A	EP, A1, 0064855 (UNILEVER NV) 17 November 1982, see the whole document --	1-2,6,9-17
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents:¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>																	
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; vertical-align: bottom;"> Date of the Actual Completion of the International Search 5th September 1991 </td> <td style="width: 50%; border-bottom: 1px solid black; vertical-align: bottom;"> Date of Mailing of this International Search Report 1991 -09- 09 </td> </tr> <tr> <td style="border-bottom: 1px solid black; vertical-align: bottom;"> International Searching Authority <div style="text-align: center;">SWEDISH PATENT OFFICE</div> </td> <td style="border-bottom: 1px solid black; vertical-align: bottom;"> Signature of Authorized Officer <div style="text-align: center;">  Yvonne Siösteen </div> </td> </tr> </table>			Date of the Actual Completion of the International Search 5th September 1991	Date of Mailing of this International Search Report 1991 -09- 09	International Searching Authority <div style="text-align: center;">SWEDISH PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: center;">  Yvonne Siösteen </div>											
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Dialog Information Services, File 351, World Patent Index, Dialog accession no. 008079604, Kanegafuchi Chem KK: "Prodn. of triglyceride by the aid of lipase - by reacting fatty acid (ester) and glycerol or partial glyceride in presence of lipase; ESTER", JP 1257485, A, 891013, 8947 (Basic)	3-5,9-17
Y	--	6
Y	WO, A1, 8901032 (NOVO INDUSTRI A/S) 9 February 1989, see page 7, line 6 - line 7 --	6
P,X	Chemical Abstracts, volume 113, no. 11, 10 September 1990, (Columbus, Ohio, US), see page 580, abstract 96171g, & JP, A, 2142484 (Manufacture of triglycerides with low water content with immobilized lipase) 31 May 1990 --	7-8,9-17
A	EP, A2, 0322213 (UNILEVER NV) 28 June 1989, see the whole document --	7-8,9-17
A	Chemical Abstracts, volume 111, no. 17, 23 October 1989, (Columbus, Ohio, US), see page 580, abstract 152188r, & JP, A, 185089 (Synthesis of triglycerides by immobilized lipase) 30 March 1989 --	7-8
A	Chemical Abstracts, volume 111, no. 25, 18 December 1989, (Columbus, Ohio, US), see page 618, abstract 230710w, & JP, A, 1108990 (Manufacture of eicosapentaenoic acid- or docosahexaenoic acid-enriched fats and oils using lipase in supercritical carbon dioxide) 26 April 1989 --	1-17
A	Dialog Information Services, File 351, World Patent Index, Dialog accession no. 007153958, Nisshin Oil Mills KK: "Polyunsaturated fatty acid glyceride prodn. by reacting the acid or its ester with glycerine in presence of heat stable lipase", JP 62091188, A, 870425, 8722 (Basic) -- -----	1-17

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this international application as follows:

See the attached sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

Lack of unity a priori

The inventions claimed are three different processes for the preparation of a triglyceride wherein all three fatty acids are polyunsaturated fatty acids. The processes are so different from each other that no technical relationship can be appreciated to be present so as to form a single general inventive concept.

The claims constitute the following three different inventions.

- 1) Claims 1-2,16,17 and partly claims 6 and 9-15 relate to a process for the preparation of a triglyceride characterized by removing water or lower alcohol during the reaction.
- 2) Claims 3-5,16,17 and partly claims 6 and 9-15 relate to a process using positionally non-specific lipase.
- 3) Claims 7-8,16,17 and partly claims 6 and 9-15 relate to a process where the lipase is immobilized on a particulate macroporous adsorbent resin of the acrylic type.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/DK 91/00100

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 91-07-31
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB-A- 2205850	88-12-21	DE-A-	3815961
		JP-A-	1063385
EP-A1- 0064855	82-11-17	AU-B-	551956
		AU-D-	8338782
		CA-A-	1178549
		US-A-	4863860
		WO-A-	82/03873
WO-A1- 8901032	89-02-09	EP-A-	0382738
		JP-T-	2504342
EP-A2- 0322213	89-06-28	AU-D-	2703488
		JP-A-	2138986